Impact of 30-day Oral Dosing with N-Acetyl-L-Cysteine on Sprague-Dawley Rat Physiology

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A number of studies have demonstrated a protective effect associated with N-acetyl-L-cysteine (NAC) against toxic chemical exposure. However, the impact of long-term oral dosing on tissue pathology has not been determined. In this study, we assessed the impact of long-term oral NAC administration on organ histopathology and tissue glutathione (GSH) and total glutathione-S-transferase (GST) activity levels in Sprague-Dawley (SD) rats. Groups of 20 SD rats (10 male, 10 female), 8 weeks of age, were dosed daily by oral gavage with deionized H_2O (negative controls) or NAC solution at a rate of 600 or 1,200 mg/kg/d for 30 days. Animals were euthanized 6 hours after treatment on study Day 30. There were no significant differences in final body weights or weekly average weight gain between treatment groups. Serum alanine aminotransferase (ALT) activities were significantly elevated ($p \leq 0.05$) in NAC-treated animals compared to controls when measured on study Day 30. Histopathologic evaluation of the liver, stomach, small intestine, liver, kidneys, spleen, thymus, and lungs revealed no lesions associated with NAC administration. When measured on study Day 30, total GST activity for kidney and skin from NAC-treated animals were increased 39-131% as compared to controls. Tissue GSH concentrations from NAC-treated animals were increased 24-81% as compared with negative controls. Further studies are needed to determine if the observed increase in tissue GSH concentration and GST activity provide a degree of chemoprotection against dermal and systemic chemical toxicants.

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The Experiments reported here were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, DHHS, Publication No (NIH) 86-23 (1996). All procedures involving live animals were approved by the WPAFB Institutional Animal Care and Use Committee (IACUC) under protocol number **F-WA-2003-0070-A.**

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Abstract
A number of studies have demonstrated a protective effect associated with N-acetyl-L-cysteine (NAC) against toxic chemical exposure. However, the impact of long-term oral dosing on tissue pathology has not been determined. In this study, we assessed the impact of long-term oral NAC administration on organ histopathology and tissue glutathione (GSH) and total glutathione-S-transferase (GST) activity levels in Sprague-Dawley (SD) rats. Groups of 20 SD rats (10 male, 10 female), 8 weeks of age, were dosed daily by oral gavage with deionized H_2O (negative controls) or NAC solution at a rate of 600 or 1,200 mg/kg/d for 30 days. Animals were euthanized 6 hours after treatment on study Day 30. There were no significant differences in final body weights or weekly average weight gain between treatment groups. Serum alanine aminotransferase (ALT) activities were significantly elevated ($p \leq 0.05$) in NAC-treated animals compared to controls when measured on study Day 30. Histopathologic evaluation of the liver, stomach, small intestine, liver, kidneys, spleen, thymus, and lungs revealed no lesions associated with NAC administration. When measured on study Day 30, total GST activity for kidney and skin from NAC-treated animals were increased 39-131% as compared to controls. Tissue GSH concentrations from NAC-treated animals were increased 24-81% as compared with negative controls. Further studies are needed to determine if the observed increase in tissue GSH concentration and GST activity provide a degree of chemoprotection against dermal and systemic chemical toxicants.

Keywords: glutathione, glutathione-S-transferase, N-acetyl-L-cysteine, oral toxicity, rats,

INTRODUCTION

N-Acetyl-L-Cysteine (NAC) is a synthetic cysteine derivative that has potential as a chemoprotective prophylaxis against toxic chemical exposures (Bobb, Arfsten, and Jederberg, 2004). NAC has been used clinically for over 25 years as an antidote to acetaminophen overdose, both in oral and intravenous forms (Smilkstein et al., 1988). NAC supplementation in laboratory animals effectively reduces the toxic effects of perfluoroisobutene (PFIB) (Lailey et al., 1991; Lailey, 1997), 2-chloroethyl ethyl sulfide (Das et al., 2003; McClintock et al., 2002), X radiation (Neal et al., 2003), and asbestos (Afaq, Abidi, and Rahman, 2000), the occurrence of urethane-associated lung tumors (De Flora et al., 1986) and bronchial epithelial thickening, hyperplasia, and markers of DNA damage in rats exposed chronically to tobacco smoke (Izzotti et al., 2001; Rogers and Jeffery, 1986). In humans, NAC is an effective chemoprotectant against toxic side-effects associated with cancer chemotherapy (Gurtoo et al., 1983; Holoye et al., 1983; Unverferth et al., 1983) and provides clinical benefit to persons with chronic obstructive pulmonary disease, probably by helping to neutralize airway H_2O_2 formation (Kasielski and Nowak, 2001). *In vitro* experiments have also demonstrated the effectiveness of NAC in reducing or eliminating toxicity in various cell types (Atkins et al., 2000; Babich and Zuckerbraun, 2001; Noh et al., 1999; Park et al., 2002; Rao and Shaha, 2002; Recchioni et al., 2002; Ryu et al., 2002).

The mechanism(s) by which NAC reduces chemical toxicity has not been fully elucidated. NAC has anti-oxidant properties (Holme et al., 1984; Pratt and Ioannides, 1985; Streeter et al., 1984) and reduces cellular oxidative damage either by directly neutralizing electrophiles or by enhancing glutathione (GSH) biosynthesis (Bobb, Arfsten, and Jederberg, 2004; De Flora, Rossi, and De Flora, 1986; Pendyala and Creaven, 1995). GSH is the major cellular antioxidant in human cells and reacts with both electrophilic and oxidizing species before they interact and possibly damage cellular components (Pompella et al., 2003). GSH is an indicator of cell function and viability with human tissue GSH levels range from 0-10 mM (Pompella et al., 2003). Certain forms of cancer, neurodegenerative, and cardiovascular diseases have been linked to GSH depletion (Pastore et al., 2003). NAC supplementation increases GSH levels in humans (De Rosa et al., 2000; Pendyala and Creaven, 1995) and animals (De Flora et al., 1985; Lailey, 1997; McLellan et al., 1995). NAC stimulates glutathione-S-transferase (GST) transcription (Xia et al., 1996), which may contribute to its anti-oxidant properties. NAC may also reduce or prevent toxicity through other mechanisms not directly involving GSH homeostasis. NAC has shown to inhibit prostaglandin synthesis (Hoffer, Baum, and Nahir, 2002), IL-8 expression (Abe et al., 2000), and may promote cell survival by stimulating NF-κB expression (Henderson et al., 1996; Xia et al., 1996).

The safety of long-term, high dose oral NAC administration in humans is an important and early consideration for determining the feasibility of using this chemical as a prophylactic chemopreventative and as a treatment for chemical over-exposure. Currently, NAC is used as a mucolytic agent and as a treatment for acetaminophen overdose (AMRM 2002). The typical oral dose for NAC as a mucolytic agent and for most other clinical indications is 600-1,500 mg/day and has been administered therapeutically by the oral route at doses as high as 2 – 4 grams daily (AMRM 2002). In cases of acetaminophen poisoning, NAC is usually administered orally with a loading dose of 140 mg/kg and 17 subsequent doses of 70 mg/kg every four hours (AMRM 2002). NAC has very low acute toxicity in humans (Gosselin, Smith, and Hodge, 2004) and is

generally well tolerated at 1 - 2 g/day. Acute side-effects reported in association with NAC administration range from gastrointestinal upset, vomiting, and fatigue to anaphylactic and allergic reactions (Bobb, Arfsten, and Jederberg, 2004; Pendyala et al., 2001; Pendyala and Creaven, 1995; Tenenbein, 1984; Ziment, 1988). Several studies have reported on the side-effects in patients administered high doses of NAC (De Flora, Grassi, and Carati, 1997; De Rosa et al., 2000; Lifshitz, Kornmehl, and Reuveni, 2000; Pendyala et al., 2001; Pendyala and Creaven, 1995; van Zandwijk et al., 2000), but the subject populations in most of these studies are patients with serious medical conditions. Therefore, the symptoms reported in these studies may not represent side-effects expected to occur in healthy populations under similar dosing regimens. We are not aware of any studies that have focused on evaluating the effects of subchronic or chronic NAC administration on clinical markers of toxicity or tissue histopathology.

In this study, we examined the effects of subchronic daily oral NAC exposure in Sprague-Dawley rats and the possible impact of daily NAC administration on tissue GSH levels and GST activities particularly in the skin. Histopathological examination focused on tissues with expected uptake or exposure to NAC (e.g., stomach, small and large intestine, liver, and kidney, the lungs, thymus, and spleen). Tissue GSH levels and total GST activity were measured after 30 days of daily NAC dosing in tissues considered primary target organs of occupational chemical exposure (e.g., skin, lungs, liver, kidney). Given the findings of the various studies published on the effect of NAC on physiology, we speculated that repeat oral NAC administration would not cause major subchronic toxicity but would increase tissue GSH and GST activity levels in all major tissues examined.

MATERIALS AND METHODS

Chemicals

N-acetyl-L-cysteine (CAS: 619-91-1, 99%) was purchased from Sigma-Aldrich Chemical Company (Saint Louis, MO). Solutions of NAC were made daily prior to dosing by dissolving NAC in warm (30°C) deionized H_2O (dH_2O) to facilitate dissolution. Both dH_2O and NAC solutions were maintained at 30°C during the time needed to administer the materials by oral gavage (approximately 30 minutes).

Animals

Four-week old male and female Sprague-Dawley CD® strain rats were purchased as a single study lot from Charles River Laboratories (Raleigh, NC). The animals were single housed upon receipt at the Wright-Patterson AFB (Ohio) vivarium and acclimated in quarantine for 4 weeks prior to beginning dosing. Subsets of animals (n=2) from the study lot were randomly selected, euthanized, and serological and histopathology samples were taken and tested for evidence of viral and bacterial infection. The study lot was sampled when they arrived at the facility and were sampled again after 2 weeks acclimation. Animals were randomly assigned to one of 3 dose groups (dH_2O, 600, or 1200 mg/kg) at the end of a 4 week acclimation period. Twenty male and 20 female rats were assigned to each dose group. Dose levels selected were approximately 12% and 24% of the single oral gavage LD50 for rats of 5,050 mg/kg (Goldenthal 1971).

Dosing and histopathology

Each day animals were weighed and dosed by oral gavage with dH_2O or NAC at a dose of 600 or 1,200 mg/kg/d 7 days a week for 30 days. Both dH_2O and NAC were administered daily in a single bolus volume of 4.8 mL/kg. After each daily dosing, the animals were observed 2 and 8 hours post-gavage for outward signs of distress and toxicity using established guidelines (OECD 2000, No.19). Food and water were made available *ad libitum* throughout the study.

Knowing that NAC concentrations in major organ systems reach a peek at 3-4 hours after a single oral dosing at a rate of 1,200 mg/kg in rats (Jung et al. 2004), all animals were sacrificed 6 hours after dosing on study Day 30. Fourteen male and 14 female animals from each dose group were anesthetized with 70% CO_2 and euthanized by exsanguination via the renal artery. Animals were exsanguinated with a 20 gauge needle and blood was collected in 5 mL syringes. The remaining animals were euthanized by intraperitoneal injection of sodium pentobarbital (SP) to allow histopathological evaluation of the lung. SP was administered at 0.25 mL/rat using a 20 gauge, 1/2-3/4 inch needle syringe. Following euthanasia, a gross examination of the thoracic and abdominal organs was performed on each animal by making a ventral midline incision extending from the level of the mandible to the pelvis and the skin reflected laterally. The thoracic cavity was further exposed by removing the ribcage along the cartilaginous junction. The gross appearance of the brain was evaluated for 5 males and 5 females per treatment group. Representative sections of stomach, liver, kidney, small and large intestine, lung, spleen, and

thymus were collected and fixed in 10% formalin. These tissues were selected for detailed study for the following reasons: a) their likely exposure to NAC via the oral route; b) a previous study in mice suggests NAC distributes to these tissues (McLellan et al., 1995); and c) clinical studies suggest that NAC may have a beneficial impact on the pulmonary and immune systems (see Introduction). Fixed tissues were processed in paraffin, sectioned, mounted, and stained with hematoxylin and eosin for histopathologic evaluation by a board certified Veterinary Pathologist at the Comparative Pathology Section of the Veterinary Sciences Branch, Brooks City-Base, Texas.

Serum chemistries

Blood samples were processed for clinical chemistry analysis following standard laboratory procedures (Stiene-Martin and Lotspeich-Steingelt, 1992). Serum chemistries were measured using a VetTest® Snap Reader (IDEXX Laboratories, Inc., Westbrooke, ME). Chemistries were measured in 100 µL of serum per animal. Serum chemistries measured were alkaline phosphatase (ALKP) activity, alanine aminotransferse (ALT) activity, urea/BUN concentration (mg/dl), creatinine concentration (mg/dl), and total bilirubin concentration (mg/dl).

Determination of tissue GST activities and total GSH concentrations

Liver, skin, kidney, and lung were harvested from individual animals, flash frozen in liquid nitrogen, and stored at -80° C. Frozen tissue (0.2 g) from individual animals was homogenized in lysis buffer (1M Tris, 5M NaCl, 2% Triton X-100, 1X PMSF) using a tissue appropriate homogenizer (e.g., glass, or Polytron). Tissue protein concentrations for each tissue were determined using a BCA protein kit (Pierce Chemical Company, Rockford, IL). Tissue protein from each animal (50 µg/tissue/animal) was collectively pooled by tissue type and NAC treatment dose level (0, 600, 1200 mg/kg). Pooled samples were then diluted with lysis buffer to a final protein concentration of 1mg/mL. Only lung tissue from animals euthanized with SP was used for lung GST activity and GSH concentration analyses.

Total GST activity of pooled tissue protein samples was determined using a GST-Tag Assay Kit (Novagen, San Diego, CA). Total GST activity was determined by monitoring the formation of glutathione-conjugated 1-chloro-2,4-dintrobenzene (DNP-SG) at 340 nm. The change in absorbance at 340 nm was monitored every 30 seconds for 5 minutes. Absorbance was monitored with a UVMax plate reader (Molecular Devices, Sunnyvale, CA). Total GST activity of each pooled sample was estimated by comparing the change in UV absorbance against a standard curve for GST activity generated with supplied positive control reagents. Enzymatic activity units (U/amount total protein) for pooled sample GST activity was calculated using Beer's Law ($A=\varepsilon lc$) where ε=extinction coefficient for DNP-SG at 340 nm ($\varepsilon_{340\ DNP\text{-}SG}$) or 10.1 $cm^{-1}mM^{-1}$ (Diah et al., 1999), l=0.3 cm, and $A=V_{max}$. GST activity was calculated using SoftMax (Molecular Devices, Sunnyvale, CA). The minimum optical density (OD) for quantification of GST activity associated with this particular assay was 0 optical density (OD); the estimated maximum OD for quantification associated with this particular assay was

approximately 200 mOD/min which equates to 2,1784 nmol/min or at least 50 times greater than our reported highest value.

Pooled sample reduced GSH concentrations were measured using a Glutathione (GSH) Detection Kit (Chemicon International, Temecula, CA). Two hundred milligrams of frozen tissue from each animal was homogenized and then pooled as described above. Pooled sample GSH concentrations were estimated by measuring the concentration of GSH present in a 10 μg aliquot from each pooled total protein sample. Ten microliters of monochlorobimane (MCB) dye was added to pooled protein samples, incubated at room temperature for 1 - 2 hours, and sample fluorescence was then measured using a SpectraMax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) and a 380/460 nm filter set. GSH concentration was estimated by comparing the fluorescence of pooled total protein with the fluorescence levels for a serial dilution of GSH positive control provided in the GSH Detection Kit. The minimum Relative Fluorescence Unit (RFU) for quantification of sample GSH concentration was 0 RFU and the estimated maximum RFU for quantification of sample GSH concentration was 20,000 RFU or at least 250,000 times greater than the highest value achieved in our analyses.

Statistical analysis

Hematology parameters and serum chemistries were expressed as mean values ± 95% confidence interval. Statistical analyses were facilitated by using SYSTAT 10.2 (Systat Inc., 2002). One-way ANOVA was used to assess the effect of NAC treatment on hematology parameters, serum chemistries, average weekly weight gain, average tissue GSH concentration, and average tissue total GST activity, as compared with those for the negative control animals (e.g., dH_2O). If statistically significant differences were found for the 3 treatment groups, the data was further analyzed using the Tukey HSD multiple comparison test with $\alpha = 0.05$ (Ott, 1988).

RESULTS

Within the first 2 weeks of the start of daily oral gavage with NAC, 1 male and 2 females from the 1,200 mg/kg/d dose group developed breathing difficulties within hours after dosing apparently as a result of mis-dosing. Symptoms were consistent with liquid present in the lungs (e.g., labored breathing, gasping, liquid exuding from the nostrils, etc), and were noticed within 1 hour after dosing. Each animal was monitored for improvement over the next 8 hours. No improvement in their clinical condition was observed and the animals were subsequently euthanized. Morphology suggestive of foreign object trauma was evident in the upper airways of all three animals at necropsy. The appearance of the lung tissue from these animals was consistent with pulmonary aspiration, indicating gavage-associated trauma. Serological assessments for foreign pathogens in the lung tissue of these animals were all negative. Data gathered on these animals during the dosing period was excluded from final analyses and tissue GSH concentrations and GST activities were not determined for these animals. No other animals were observed to have gavage-associated trauma at necropsy.

No clinical symptomology consistent with chemical toxicity were observed at any time in NAC-treated animals. No other animals died during the study. Anthropomorphic parameters did not differ significantly for the 3 treatment groups (Table 1).

TABLE 1

Comparison of anthropomorphic parameters for rats given 0, 600 mg/kg, or 1,200 mg/kg NAC by oral gavage for 30 days (± 95% C.I.)

Parameter	Dose Group		
	0	600 mg/kg	1,200 mg/kg
	Male rats		
N	10	10	9
Final average body weight, grams	435.7 ± 20.4	416.8 ± 25.5	414.9 ± 16.9
Average weekly weight gain, grams	3.7 ± 2.5	3.1 ± 2.0	3.1 ± 1.8
	Female rats		
N	10	10	8
Final average body weight, grams	268.1 ± 13.3	268.6 ± 14.9	274.4 ± 17.6
Average weekly weight gain, grams	1.1 ± 1.5	1.0 ± 1.6	1.1 ± 2.0

Average serum ALT activities were increased significantly for animals treated for 30 days with 600 or 1200 mg/kg NAC as compared with the average for negative controls (Table 2). There were no significant differences found for the 3 treatment groups for serum ALKP activity, urea, creatinine, or total bilirubin when analyzed by one-way ANOVA ($p \leq 0.05$).

TABLE 2

Study group serum chemistry results (± 95% confidence interval)

Serum chemistry	Limits of Detection	dH_2O	600 mg/kg	1200 mg/kg
ALKP (Units/Liter)	10 - 2,000	191 ± 53	150 ± 73	145 ± 54
ALT (Units/Liter)	10 - 1,000	43 ± 5	171 ± 65[a]	89 ± 47[a]
Urea or BUN (mg/dl)	0 - 130	17 ± 1	13 ± 3	15 ± 3
Creatinine(mg/dl)	0 - 13.6	0.5 ± 0.05	0.9 ± 0.3	0.5 ± 0.2
Total bilirubin (mg/dl)	0 – 27.9	0.4 ± 0.1	0.2 ± 0.06	0.3 ± 0.1

Significantly different from control value: [a] $p \leq 0.05$ (t-test)

On study Day 30, all animals were euthanized and full necropsies were performed with particular focus on gross and histopathological changes in the stomach, small intestine, liver, kidneys, spleen, thymus, and lungs. No evidence of chemical toxicity or irritation was found in the tissues examined and no tumors or other histopathological anomalies were found in any study animals at necropsy. For a majority (10/17) of the animals treated with 1,200 mg/kg NAC, significant distention of the small and large intestines and a foul sulfur odor similar to the smell produced by warming NAC in warm (e.g., 30°C) dH_2O (see Methods) was evident upon opening of the visceral cavity at necropsy on study Day 30. No bleeding or rupture of the GI tract was observed for animals with significant GI distention.

Both total GST activity and GSH concentration were increased significantly for kidney and skin homogenates isolated from animals treated with NAC as compared with those isolated for negative controls (Table 3 and 4). GSH concentrations were significantly decreased in liver and lung tissue homogenates isolated from animals treated with NAC as compared with those from tissue homogenates isolated from negative controls (Table 4).

TABLE 3

Comparison of average total GST activity measured in various tissues from rats treated with dH_2O versus NAC for 30 days by oral gavage

Tissue	Dose group	Average activity (Δnmol/min ± 95% C.I.)	% change compared to dH_2O
Liver	dH_2O	400.0 ± 33	
	600 mg/kg	391.8 ± 27	(-) 2
	1,200 mg/kg	421.9 ± 112	6
Kidney [a]	dH_2O	110.5 ± 12	
	600 mg/kg	181.3 ± 19	64
	1,200 mg/kg	255.2 ± 37	131
Lung	dH_2O	399.5 ± 148	
	600 mg/kg	409.9 ± 19	3
	1,200 mg/kg	416.6 ± 101	4
Skin [a]	dH_2O	66.7 ± 7	
	600 mg/kg	92.7 ± 10	40
	1,200 mg/kg	151.2 ± 16	127

Significant difference between groups: [a] $p \leq 0.05$ (ANOVA)

TABLE 4

Comparison of average GSH concentrations measured in various tissues from rats treated with dH_2O versus NAC for 30 days by oral gavage

Tissue	Dose group	Average concentration (μM/μg total protein ± 95% C.I.)	% change compared to H_2O
Liver [a]	dH_2O	8.2 ± 0.6	
	600 mg/kg	6.6 ± 0.3	(-)20
	1,200 mg/kg	7.2 ± 0.6	(-)14
Kidney [a]	dH_2O	2.6 ± 0.1	
	600 mg/kg	4.7 ± 0.2	80
	1,200 mg/kg	3.8 ± 0.1	46
Lung [a]	dH_2O	4.4 ± 0.1	
	600 mg/kg	3.4 ± 0.1	(-)23
	1,200 mg/kg	3.8 ± 0.1	(-)14
Skin [a]	dH_2O	4.2 ± 0.0	
	600 mg/kg	5.2 ± 0.1	24
	1,200 mg/kg	7.2 ± 0.2	71

Significant difference between groups: [a] $p \leq 0.05$ (ANOVA)

DISCUSSION

Gastrointestinal discomfort, nausea, dyspepsia, diarrhea, and other clinical symptoms central to the gastrointestinal system are common complaints from patients taking or receiving NAC (Holdiness, 1991; Pendyala et al., 2001; Pendyala and Creaven, 1995; van Zandwijk et al., 2000). Daily inspection did not find evidence of increased frequency of loose stools among NAC-treated animals. Significant gas production and bloating was present at necropsy of animals treated with 1,200 mg/kg NAC suggesting that toxicity may have been occurring in the stomach and small and large intestine after 30 days of daily exposure to NAC. It is difficult to know whether these postmortem findings were evidence of a negative impact on the well-being of these animals over the 30-day study period. No clinical signs of toxicity were apparent in NAC-treated animals at any time during the 30-day dosing period and the average weight gain of these animals over the study period did not differ significantly from the average weight gain for negative controls. Histopathological evaluation of the major digestive tract organs (e.g., stomach, small and large intestine, liver) found no evidence of toxic response in these organs (e.g., irritation, degeneration, necrosis) in NAC-treated animals. Although serum ALT activities were elevated in NAC-treated animals, microscopic evaluation of liver sections from NAC-treated animals did not find evidence of tissue changes suggestive of toxicity.

The effect of daily oral dosing with NAC was evaluated in the lungs. NAC is known to have anti-mucolytic properties (Lailey et al., 1991; Lailey, 1997; Webb, 1962) and the lung is a known sight of NAC distribution and uptake (Jung et al., 2004; McLellan et al., 1995). Bronchospasm is a potential complication associated with administration of high doses of NAC (Dorsch, Auch, and Powerlowicz, 1987; Ho and Beilin, 1983; Ziment, 1988). Our analyses did not find evidence of histopathological changes occurring in the rat lung as a result of daily oral administration of NAC. However, our study did not evaluate the lung function of NAC-treated animals and it is possible that NAC administration may have had a subtle effect on lung function, absent of recognizable histopathological changes in the lung but not detectable during daily observation of the gross clinical condition and behavior of NAC-treated animals.

Previous studies suggest that there is a correlation between NAC administration and enhanced immune response. NAC treatment is correlated with an increase in T cell immunity (Breithaupt et al., 1996; Droge, Eck, and Mihm, 1992; Wu, Levy, and Black, 1989) and may provide therapeutic benefit for a wide range of diseases (Beloqui et al., 1993; Bengtsson et al., 2001; De Flora, Grassi, and Carati, 1997; Walters et al., 1986) possibly by modulating immune response. Intravenous administration of large doses of NAC as an antidote for acetaminophen poisoning can cause anaphylactic responses in sensitive individuals (Lifshitz, Kornmehl, and Reuveni, 2000; Reynard, Riley, and Walker, 1992) most likely due to histamine release (Lifshitz, Kornmehl, and Reuveni, 2000). Allergic reactions in the form of rash and angiodema have been reported in persons receiving NAC intravenously (Tenenbein, 1984). In this study, we found no evidence of an effect of repeat administration of NAC on the gross appearance or size of the thymus or spleen. Also, there were no changes detected that would suggest NAC treatment had an impact on the histology of these tissues. White blood cell (WBC) counts from NAC-treated animals were not significantly increased as compared with controls (data not shown); a significant increase in WBC count is a marker for immune stimulation (Miller, 1969). We did

not assess the immune function/response or T-cell differentials in NAC-treated animals, therefore the effect of NAC treatment on these parameters could not be determined.

Although the liver may play a significant role in NAC metabolism, several studies including our own have shown that liver GSH levels are not significantly increased following NAC (De Flora et al., 1985; McLellan et al., 1995; Pratt and Ioannides, 1985), or oral GSH (Aw, Wierzbicka, and Jones, 1991) administration. NAC is a proven effective countermeasure for liver toxicity as a result of acetaminophen overdose (Corcoran and Wong, 1986; Lauterburg, Corcoran, and Mitchell, 1983) and this has lead to the hypothesis that NAC is chemoprotective because it boosts GSH biosynthesis in the liver. Alternatively, NAC may serve as a supplemental source of cysteine for GSH synthesis when hepatocyte GSH levels become depleted through GST-mediated conjugation or direct interaction with the acetaminophen metabolite N-acetyl-*p*-benzoquinoneimine (NAB). NAC may also substitute for intracellular GSH or increase cellular cysteine levels in the liver. GSH levels in the liver are augmented by circulating GSH in plasma (Aw, Wierzbicka, and Jones, 1991), and this may be a possible explanation as to why GSH levels do not become elevated in the liver after NAC administration. Our finding that subchronic NAC administration did not increase GSH levels in the rat liver may be because there was no concurrent exposure to GSH-depleting substances in this study (e.g., NAB). As a result, liver GSH levels did not change significantly as compared with negative controls. It is possible that the timing of the sacrifice for animals at Day 30 was not soon enough after NAC administration to detect changes in GSH concentrations in the liver. However, studies in our laboratory of ^{14}C-NAC distribution in rats indicate that the level of ^{14}C cysteine moiety remains significantly elevated in the liver for up to 12 hours post-dose after oral gavage with radiolabeled ^{14}C-NAC given at a rate of 1,200 mg/kg and 1 μCi per animal (Jung et al. 2004). This suggests that NAC or cysteine levels are elevated in the liver after a single oral dose without a concomitant increase in intracellular GSH, but this cannot be verified without characterizing liver GSH levels at intervals between 0.5 – 12 hours post-dosing. Studies are currently ongoing in our laboratory to characterize the effect of a single NAC administration by oral gavage on NAC, GSH, and cysteine levels in major organs during the 0.5-12 hours post-dosing time period.

We have shown in this study that NAC administration by the oral route significantly increases skin GSH concentrations in a dose-dependent manner in rats. We are not aware of any studies that have demonstrated that oral administration of NAC can increase skin GSH concentrations. A previous study has shown that NAC or its radiolabeled cysteine moiety distributes to the hair follicles of mice injected with radiolabeled NAC (McLellan et al., 1995). Our finding that NAC administration by oral gavage increases GSH levels in the rat kidney correlates with previous reports that NAC is an important site of cysteine uptake (McLellan et al., 1995) and that NAC administration diminishes kidney toxicity associated with chemical exposure (Alonso et al., 2004; Imberti et al., 1990; Na, Jeong, and Lim, 1992; Park et al., 2002). These findings in combination with the results reported in this study suggest that NAC may reduce or prevent chemically-related kidney toxicity by a GSH/GST-mediated mechanism. The deacetylation of NAC to cysteine is believed to be an important step in the formation of NAC-derived GSH. Both rodent and human kidney have significant deacetylating activity via the enzyme acylase I (Durand et al., 2003; Yamauchi et al., 2002), which is localized in the proximal tubules (Yamauchi et al., 2002). The deacetylating activity of the rat kidney is 182-190% higher

than that of the liver (Yamauchi et al., 2002), and may partly explain our findings in relation to the lower GSH concentrations that occurred in liver as compared to the kidney.

GSH is thought to play an important role in protecting the lungs from electrophilic and oxidative damage (Cantin et al., 1987; Rogers and Jeffery, 1986). Our finding that repeat oral administration of NAC to rats does not increase lung GSH content is consistent with findings of Lailey (1997) and Lailey et al. (1991). However, lung cysteine levels were found to be increased 150% following intraperitoneal injection of NAC (Lailey et al., 1991). It is not clear why oral NAC administration does not increase lung GSH content but it is possible that intraperitoneal injection has different absorption routes that lead more directly to the lungs. High circulating levels of NAC, cysteine and GSH in blood may provide abundant cysteine to enable the lung to enhance its defense when challenged with toxic exposures (Lailey, 1997). We found that cysteine and NAC levels remain significantly elevated in serum of rats for up to 24 hours after oral administration of NAC. However, NAC and cysteine levels were not significantly elevated in circulating red blood cells when assessed at 1 – 24 hours post-administration (Jung et al. 2004).

A unique finding not previously reported was the finding that total GST activity in kidney and in skin was increased significantly after daily oral gavage with NAC for 30 days. Previous studies reported transient increases in total GST activity of ≥30% in peripheral blood lymphocytes occurred in healthy volunteer smokers who took 1,200 mg NAC per day by mouth (Pendyala et al., 2001). GST is responsible for conjugating GSH to molecules targeted for export from the cell. Reasons as to why antioxidants induce GST protein transcription (Xia et al., 1996) and GST activity are not known. Evidence suggests that GST may share regulatory elements with enzymes involved in cellular GSH homeostasis (γ-glutamylcysteine synthase) or possibly post-GST conjugation functions (e.g., mercapturic acid biosynthesis). Induction of GST transcription by antioxidants such as NAC is most likely mediated by activator protein-1 (AP1) (Li and Jaiswal, 1992; Pinkus, Bergelson, and Daniel, 1993; Xia et al., 1996) and nuclear factor-κB (NF-κB) binding sites (Xia et al., 1991).

In conclusion, administration of NAC at a rate of 600 mg/kg/d for 30 days did not produce evidence of clinical toxicity whereas administration of NAC at a dose rate of 1,200 mg/kg/d for 30 days was associated with bloating and excess gas production in the stomachs, small intestines, and large intestines at necropsy on study Day 30. Serum ALT activities were increased in animals treated with NAC at a rate of 600 or 1,200 mg/kg/d. However, histological evaluation of stomach, liver, kidney, small and large intestine, lung, spleen, and thymus sections did not find evidence of chemical toxicity or irritation. The significant increases in both GSH concentration and GST activity of skin and kidney after 30 days of oral NAC administration suggests that daily NAC intake not only enhances GSH synthesis in these tissues, but may also be chemoprotective by enhancing detoxification via GSH conjugation. Further studies are needed to determine if the increases in tissue GSH concentration and GST activity measured in this study translate to a reduction in the severity or prevention of toxic pathology expected as a result of exposure to proven dermal and systemic chemical toxicants. Future work will also investigate the threshold dose level and number of dosing days necessary to significantly elevate skin GSH and cysteine levels.

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